A COMPARATIVE STUDY OF THERMOINACTIVATION OF HEMAGLUTINATING AND INFECTIOUS ACTIVITY OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

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Any extensive study of the epidemiology of arbovirus infections requires that we develop and perfect methods for the production of infection antigens. At the present time there are two generally accepted methods for the production of noninfectious preparations of the hemagglutinins of the arboviruses — treatment with beta-propriolactone and treatment with heat [7]. In the present study an attempt is made to establish the relationship between the mechanisms of thermoinactivation of the infectious and hemagglutinating activity of one representative of the arboviruses, namely the virus of Venezuelan equine encephalomyelitis (VEE virus), and also to justify theoretically some method for the thermal production of non-infectious hemagglutinins.

Material and methods. The VEE virus used had been put through 28 passages in a culture of chicken embryo fibroblasts (CEF). We employed a culture virus-containing liquid (medium No 199, with 2% heated cattle serum), the initial activity of which amounted to 9-9.5 lg BSU/ml and 9-10 $\log_2$ HAU/ml.

The cell culture was prepared in the usual manner [1]. Hemagglutinating activity was determined with use of the Clarke-Casals method [10]. The plaque-over-agar method was used in titrating the infectious activity of the virus [11].

An ultrathermostat was used to heat the virus-containing suspension, following the generally used method [6], with slow titration of the infectious and hemagglutinating activity.

Results. Thermoactivation of the hemagglutinating activity of the VEE virus. We produced inactivation of VEE virus hemagglutins in the temperature range from 50-60°C. At temperatures below 50°, the limited accuracy of the method did not permit a reliable estimate of the dynamics of inactivation during the first 1-3 hours of heating; and at temperatures above 60° the inactivation proceeded too rapidly to permit adequate conduct of the experiments.

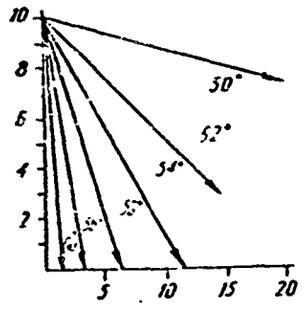

Figure 1. Inactivation of VEE virus hemagglutinins at various temperatures.

Virus activit. is plotted on the y-axis (in $\log_2$HAU/ml), time of heating (in min.) on the x-axis.

The data illustrated in Figure 1 above support the conclusion that inactivation of the hemagglutins of the VEE virus in the indicated medium proceeds at a rate proportional to the heating temperature. The dynamics of inactivation at various temperatures are indicated schematically here; the indicated figures are the results of averaging five parallel tests. By reason of the presence of virus particles in the medium which are soluble and tend to "mask" the hemagglutinins, the picture presented here is to some degree unrealistic.

On the basis of the dynamics of inactivation arrived at, we computed the logarithms of the inactivation rate constants ($\lg k_{in}$) as given below in Table 1. On a parallel basis, we determined the values for $\lg k_{in}$ for the infectious activity of the initial virus strain, using a 4 to 60° temperarange.

TABLE 1

Rate Constants of Inactivation of VEE Virus Hemagglutinins

Temperature (°)	$-\log k_{in}$
50	2.33
52	1.78
54	1.54
56	1.24
58	1.01
60	0.65

TABLE 2

Thermodynamic Characteristics of the Process of Thermoinactivation of the VEE Virus

Biol. activity	Activation enthalpy (ccal/mol)	Activation entropy (enthropy units)
Hemagglutinins	68.95	152.27
Infectivity:		
"Protein type"	75.8	162.8
"Nucleic type"	26.0	3.98

Relationship between inactivation of VEE virus and temperature.

Using the values obtained for inactivation rate constants, we constructed graphs for the Arrhenius relationship between lg k_{in} and inverse absolute temperature (1/T, Figure 2). In Figure 2-A the Arrhenius relationship reflects the process of inactivation of infectiousness of VEE virus within the temperature range from 4 to 60°; in Figure 2-B it reflects the process of inactivation of hemagglutinating activity. Upon superimposition, the graph reflecting hemagglutinin inactivation completely coincides with steep, high-temperature portion of the infectivity curve.

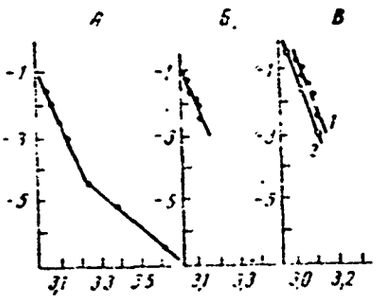

Figure 2. Arrhenius relationship of the process of inactivation of the VEE virus.

A - inactivation of infectivity; B - inactivation of hemagglutinins of initial (1) and thermostable (2) strains. Here, as in Figure 3, the logarithms of the inactivation constants are plotted on the y-axis, and the inverse absolute temperatures (X1,000) on the x-axis.

Using Eyring's formula [12], we calculated the values of enthalpy (ΔH) and entropy (ΔS) of activation of the process of thermoinactivation of hemagglutinins, and also of the steep and the smooth portions of the infectivity inactivation curve. These data are given in Table 2 above.

The values of the enthalpy and entropy of activation of the

hemagglutinin thermoinactivation process coincide well with the values of ΔH and ΔS of infectivity activation of the VEE virus of the high-temperature, "protein" [3] type.

TABLE 3

Thermodynamic Characteristics of Inactivation of the Initial and the Thermostable Variants of the VEE Virus (Temperature Range, 50-60°)

VEE virus	Biological Activity	Enthalpy (ccal/mol)	Entropy (entr. units)
Initial	Hemagglutinins	68.95	152.27
	Infectivity	73.8	162.8
Thermostable	Hemagglutinins	112.2	271.5
	Infectivity	101.9	241.08

Inactivation of the thermostable variant of the VEE virus. The present writers reported earlier on separation of the thermostable variant of the VEE virus, and on some of its properties [4, 5]. We also determined the dynamics of hemagglutinin inactivation and infectivity during heating, and calculated the thermodynamic characteristics of the process, which determine its progress at high temperatures (50-60°C). These data are given in Table 3 below. In Figure 2-c is shown the Arrhenius relationship between the processes of hemagglutinin inactivation in two types of virus, the initial and thermostable. The results here show that increase in heat-resistance in the 50-60° range for the thermo-

stable variant is also characteristic for infective, just as it is for hemagglutinating, activity.

Discussion. As is well known, inactivation of viruses at various temperatures is determined by two mechanisms [3, 9]. At low temperatures, change in infectious activity occurs as a result of destruction of viral nucleic acids: in other words, it follows the "nucleic" type, and on the Arrhenius graph is reflected by the position and the slope of the smooth portion of the graph.

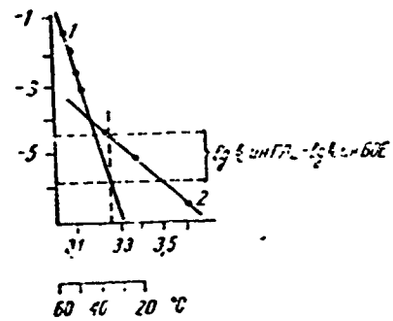

Figure 3. Diagram illustrating the Arrhenius relationship of the mechanism of the thermal separation of infectious and hemagglutinating activity of the VEE virus.

1 - "protein" type; 2 - "nucleic" type

High-temperature inactivation of infectivity is associated with primary change in the viral proteins: that is, it follows the "protein" type. On the Arrhenius curve, this process is characterized by the slope angle and position of the steep portion of the curve. Every type of in-

activation is distinguished by definite thermodynamic parameters which are peculiar to it [3].

TABLE 4

Inactivation of Infectious and Hemagglutinating Activity of VEE and Sindbis Viruses (36°)

Type of virus	Activity	Incubation time (in days)				$-\lg k_{in}$
		0	3	5	7	
VEE	Infectious (lg BSU/ml)	9.5	2.3	0.7	0	4.43
	Hemagglutinating ($\log_2$ HAU/ml)	10.3	10.0	10.0	9.4	5.6
Sindbis	Infectious (lg BSU/ml)	9.4	0.6	0	0	4.12
	Hemagglutinating ($\log_2$ HAU/ml)	11.0	10.1	10.0	10.0	5.3

The results given above show that inactivation of hemagglutinins proceeds according to the type of destruction of viral proteins, the type being determined, in all probability, by disruption of the hydrogen bonds within the molecules of the virions, which results in their denaturing [2]. Since stability at high temperature is conditioned by the properties of the viral proteins, it would be natural to expect increased stability of

the hemagglutinins of the thermostable varient of the VEE virus, as compared with the initial virus strain; and this conclusion is indeed supported by the data which we collected.

Considering that the two types of inactivation of the VEE virus which are reflected in the two-compenent Arrhenius curve are determined by the accuracy of the crossing point of the curve [3], it would appear possible to extrapolate that portion of the curve characterizing hemagglutinin inactivation, extending it up to the temperature at which, usually, noninfectious hemagglutinins are obtained (Figure 3). Here the curve distinctly demonstrates the fact that at 33-36°, there is at least 10 times slower inactivation of hemagglutinins than there is inactivation of infectivity.

To confirm this contention, we ran a series of special experiments on a model of two RNA-containing viruses — VEE and Sindbis. In Table 4 above are shown averaged results of parallel tests to determine inactivation of infectious and hemagglutinating activity of these two viruses at 36°C in an accumulation medium (medium No. 199 with 2% cattle serum). For both viruses, the difference in hemagglutinin and infectivity inactivation rate constants is pretty close the theoretical value ($\Delta \lg k_{in}$ = = 1.17 for the VEE virus, and = 1.18 for the Sindbis virus,. These figures, together with accumulated experimental experience in obtaining noninfectious hemagglutinins with the use of heat [7], entirely support the proposed theoretical model.

Determination of the thermodynamic characteristics and structure of the Arrhenius relationship for the thermoinactivation process makes

possible the following: 1) an explanation of the mechanism of separation, with use of heat, of infectious and hemagglutinating activity; 2) to determine, with a high degree of reliability, the optimal conditions for obtaining, with the use of heat, noninfectious hemagglutinins of various viruses; and 3) to assert that a thermostable variant of the VEE virus is more suitable for obtaining noninfectious hemagglutinins with the use of heat, since increase in the slope of the Arrhenius curve indicates a greater stability of the hemagglutinins, and also more substantial differencesin the inactivation rates of hemagglutinins and infectivity at a given working temperature.

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Thermodynamical values and Arrenius relationship of the process of thermoinactivation of Venezuelan equine encephalomyelitis virus hemagglutinins were determined. Enthalpy and entropy of activation were found to be 68.96 kcal/mol and 152.27 E.U., respectively, for the original strain and 112.2 kcal/mol and 271.5 E.U. for the thermostable variant of the virus. Parameters of the process correlated well with the characteristics of inactivation of the infectivity of the virus by the "proton" type. The possibility of using thermodynamical characteristics of the thermoinactivation process for determination of optimal conditions for most separation of the infectious and hemagglutinating activity of the virus is discussed.